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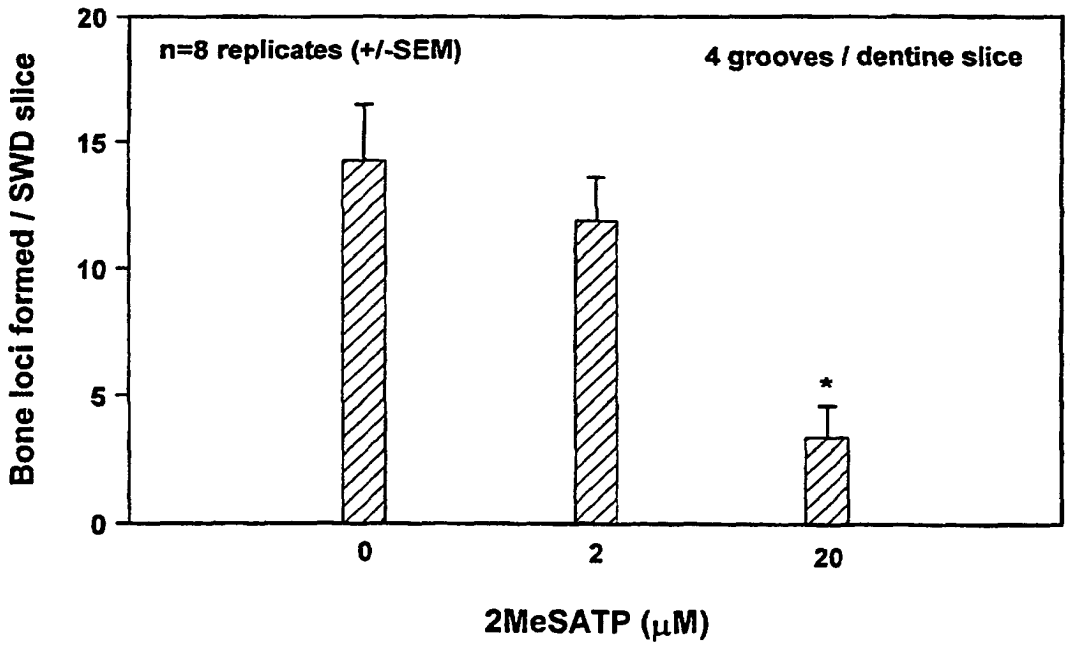

(57) Abstract

Osteoblasts are cultured on a surface having a topography adapted to promote or enhance formation of bone, thus directing bone formation at predictable and desired sites. In an assay for the effects of a drug on bone formation, osteoblasts are cultured in a groove approximately 350 microns wide and 200 microns deep. The bone formation surface can be provided on a culture plate or on a prosthetic implant or transplant.

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SITE-DIRECTED BONE FORMATION

This invention relates to site-directed bone formation, both in vivo and in vitro.

In particular, this invention relates to a method of bone formation in vitro and to an assay for the effect of various substances on such bone formation. The invention relates also to a method of promoting or enabling bone formation in a localised area of a surface, in particular the surface of a prosthetic device or implant or graft. The invention further relates to a method of modifying a surface to promote or enable formation thereon of bone. The invention relates additionally to prosthetic devices, implants, grafts, culture surfaces and other such surfaces adapted to promote or enable bone formation thereon.

There exists, in this art, known problems with the design of culture surfaces and surfaces on prosthetic devices, implants and grafts such that bone formation on these surfaces firstly occurs and secondly occurs in a predictable way and in a predictable location. There exists also the problem that the formed bone on existing such surfaces in vitro may not in fact be true bone and does not therefore present an accurate or useful model for in vivo bone formation.

In the field of orthopaedic implants, most are MMA-PMMA cemented, with the advantages of instant stability, but the disadvantages of tissue death from heat production during polymerisation and of a foreign body, immune system response. Direct bonding of bone to a porous metal or HA (hydroxyapatite)-coated implants (Stephenson et al 1992) gives rise to difficulties in removing the bone-bonded device if revision is required for any reason. The rapidity and quality of the formation of a union of bone with an implant might be improved if their relationship were better ensured by a rapid healing process in the first instance, but rapid and site-predictable union is not achievable with present implants.

Approaches to the 'shaping' of the skeletal implant surface intended for non-cemented

union have been based upon the possibilities in metallurgical and/or ceramic technology rather than a precise knowledge of requirements. A large literature documents ingrowth of bone into pores in the surfaces of implants with a finer scale of relief, but the time course of healing is unknown. With the macro-relief produced by machining metal into a helical screw thread, or into a series of ridges of millimetre size, cartilage may form on the multiple load bearing (transverse) surfaces of such devices, with new bone ingrowth into the large voids not contacting the implant (Revell, 1986). Both of these latter situations are possibly undesirable.

Many attempts have been made to devise and exploit porous materials or porous surface coatings to secure good mechanical interlocking of an implant to bone tissue (such as: Schliephake H, Neukam FW, Klosa D (1991) Influence of pore dimensions on bone ingrowth into porous hydroxyapatite blocks used as bone graft substitutes. A histometric study. Int J Oral Maxillofac Surg 20:53-58; 1991, or Dalton JE, Cook SD, Thomas KA, Kay JF (1995) The effect of operative fit and hydroxyapatite coating on the mechanical and biological response to porous implants. J Bone Joint Surg Am 77:97-110). These materials include porous titanium, cobalt-base alloys, Cr-Co-Mo alloys, carbon, high density polyethylene and alumina, calcium aluminate and calcium phosphate ('hydroxyapatite') ceramics. Coral partially converted to hydroxyapatite has been used as a graft into which bone forms. (PMMA bone cement may also be porous, but does not normally sustain a direct union to bone, as described by Al Saffar N, Revell PA (1992) in Interleukin-1 production by activated macrophages surrounding loosened orthopaedic implants: a potential role in osteolysis. Brit J Rheumatol 1994 33:309-316). With all such approaches, it has not been the aim to determine, nor is it known, whether bone grows out from the implant to meet the surrounding bone tissue or from the bone tissue towards the implant. In no presently available system can the site of bone formation be specified topographically.

Osteoporosis and osteoarthritis are both widespread diseases with a huge economic cost to society. Any improvement in treatment modalities for this large sector of the ageing population will be of great importance to improving the quality of life for the sufferers and their minders and relatives.

An existing assay for bone formation in vitro involves measurement of bone nodule formation. Some observers now consider that the bone nodules formed in this assay do not represent true bone, therefore raising a question mark over the validity of this assay. Further, the formation of nodules in this assay occurs in unpredictable locations dispersed throughout the assay medium. Other comments on the importance of obtaining true in vitro bone appear in an editorial in Virchows Archiv 426:103–105 (1995) by Andreas Schulz.

The present invention consequently has various objectives. These include site-directed bone formation, both in vitro and in vivo. It is another object to provide in vitro bone formation of true bone. A further objective is to provide an accurate and reliable assay for the effect of a substance on in vitro bone formation. Still further objectives relate to providing bone formation surfaces on prosthetic devices, implants and grafts.

The objectives also include to develop improved contours for skeletal implant surfaces, the facing bone surfaces, and bone grafts. New types of implant surface profile, and the micro-machining of bone grafts and graft sites, may lead to faster and better healing.

The invention thus relates to modification of surface topography to promote or enable bone formation thereon.

Accordingly, a first aspect of the invention provides a method of promoting or enabling bone formation in vitro comprising culturing osteoblasts on a surface having topographical features that direct said formation in a localised area.

In an embodiment of the invention, a first surface portion is depressed in relation to an adjacent, raised surface portion, forming for example a substantially L or V or U or semi-circular shaped junction between the respective depressed and raised portions. A culture surface according to the invention can thus include features selected from a ridge, multiple ridges having a saw tooth like cross section, a step, a series of steps,

a trench, trenches, a groove, grooves and combinations of the aforementioned surface features. These features are optionally provided on culture apparatus such as a culture dish or on a separate surface suitable to be placed inside or attached to culture apparatus, for example a plate to go inside a culture dish.

The height of the raised surface portion, such as a ridge, in relation to the depressed surface portion is preferably 100 microns or greater. The maximum height is in practice usually limited by the width of the culture surface at that point. With a culture dish having a base of 2-3 millimetres thickness, the height difference between adjacent raised and depressed surface portions formed on the base is conveniently 100-1000 microns, more commonly 150-500 microns.

Another embodiment of the invention comprises carrying out the culture on a surface comprising one or a plurality of pits or grooves adapted to promote or enable bone formation therein. Suitable pits, grooves or depressions include those having a substantially U-shaped, rectangular or semi-circular cross-section. It is preferred that the width of the pits, grooves or depressions is in the range 150-500 microns, more preferably between 250 and 450 microns. It is further preferred that the depressions, pits or grooves have a depth in the range of 50-500 microns, more preferably between 150 and 400 microns, and most preferably 200-300 microns.

The width of the grooves, pits or depressions can be greater than 500 microns, in which case bone is usually seen first on opposing sides and subsequently the areas of bone enlarge towards the centre of the groove, pit or depression, eventually covering the whole width if culture is maintained for a sufficient duration.

In a specific embodiment of the invention, described below, bone growth is seen in a groove approximately 300 microns wide, and of variable depth. In another specific embodiment, also described below, bone is grown in a groove about 350 microns wide and about 200 microns deep.

It is optional that the surface of a groove has small surface abrasions, preferably

substantially in the direction of the groove or transverse thereto. Grooves cut into a surface such as the base of a tissue culture plate typically contain surface abrasions as an artefact of the cutting process, but bone growth also occurs with smooth surfaces, for example the right angle at the edge–wall of a plastic culture dish acts as a nidus for bone formation. Grooves can be formed directly in the base of a tissue culture vessel or on aseptic surfaces that are added to the culture vessel. Alternatively, ridges of equivalent size to the grooves support bone growth in the angle with the flat surface of, say, a culture dish.

It has thus surprisingly and advantageously been found that by providing surface topographies for in vitro culture of osteoblasts, according to the present invention, bone formation occurs preferentially at predictable sites. The culture methods are suitably carried out with osteoprogenitor cells of whatever origin, use of the surface topographies of the invention resulting in formation of bone by osteoblasts derived from the osteoprogenitor cells.

Further, analysis of the bone formed shows that it closely resembles true bone, thereby both providing for in vitro formation of true bone and for representative in vitro assays of bone formation, hitherto unattainable in the art. In detail, bone was examined by 3–D light microscopy of stained specimens and by confocal light microscopy (used in both reflected and fluorescence modes, checking for the normal autofluorescence shown by bone) of unstained specimens. In both cases, examination confirmed that the bone obtained is morphologically bone. Independent examination using conventional light microscopy and histology, carried out at the Anatomy Department, Liverpool University, UK also confirmed the bone to be true bone.

Other preferred surface topographies are apparent from preferred embodiments and specific examples described herein. In a particular embodiment, the method comprises culturing osteoblasts on a culture surface comprising a series or a plurality of spaced grooves. Bone formation occurs preferentially in and along the grooves. If and when the groove depth varies, bone formation typically occurs to a greater or earlier extent in deeper parts of the groove or in deeper grooves.

Bone growth is typically measured by a variety of different methods, such as (i) counting the number of loci at which bone growth is detectable, (ii) measuring the length of bone formed on a substrate, for example along a groove on a culture surface, (iii) measuring the area of bone formed on each substrate, (iv) recording the day on which bone is first detectable and (v) measuring the volume of bone formed.

In specific embodiments of the invention, described below, bone is grown in culture dishes 35mm in diameter, having a surface area of about 960mm^2. These dishes are seeded with 50,000–400,000 osteoblasts, more often 200,000–300,000 osteoblasts, so the density of seeding is in the range 50–420 osteoblasts per mm^2, more commonly 210–315 per mm^2. At this latter seeding density, bone growth is typically first detectable about 16 days from the start of the culture. Seeding densities both higher and lower than these specified amounts have also been used. When the initial osteoblast density is increased above the specified density the duration before bone growth is first detected is slightly decreased though not in proportion to the increase in osteoblast density. When the initial seeding density is decreased then the time until bone is first detected is increased, roughly in proportion. In all cases, the nature of bone formed appears exactly similar.

It is possible, in a further particular embodiment of the invention, to exploit preferential or earlier growth of bone in deeper grooves of similar width, by providing on a culture surface a groove or a series of grooves each of similar width and having a graduated depth that increases along the length of the groove. Such a culture surface is of particular use as the extent of bone formation can be determined by measuring the length of groove in which bone formation has occurred.

Accordingly, a second aspect of the invention provides a method of assaying a substance for its effect on bone formation by osteoblasts, comprising:–

(a) culturing osteoblasts in the presence of the substance and according to the culture methods of the first aspect of the invention.

For a determination of the effect of the substance, bone formation is then compared with bone formation in the absence of the substance. In a typical assay, a number of concentrations of the substance are tested.

The assay of the invention offers the advantage that bone formation is in predictable locations and is consequently more readily measured, producing a more reliable assay. In a specific embodiment of the invention, hereinafter to be described, the assay comprises culturing osteoblasts on a culture surface comprising a series of substantially parallel grooves of even (or graduated) depth, measuring the length of confluent bone formation in each (or every) groove at set time points and expressing the degree of bone formation as a ratio of this length compared to the length of confluent bone formation in a similar culture carried out in the absence of the assayed substance. This assay thus provides an accurate and easy-to-use means of assaying bone formation for a substance of interest.

In the case that the depth of the groove is graduated, it is preferred that the groove depth varies evenly up to at least 200 or 300 microns deep.

Measurement of bone formation can be achieved with the culture living and unstained, though it is preferable to stain or mark formed bone for ease of its observation. One suitable method is, at the end of the culture period, to fix the culture using an alcohol solution and subsequently to stain bone formed in culture using a coloured dye such as alizarin red S (CI S8005). Following staining, observation of the length of groove occupied by bone is considerably facilitated.

Suitable materials for the culture surface include dentine, bone or other mineralised tissue, plastics material, alloys, titanium alloys, glass or other inert material that substantially does not interfere with osteoblast culture.

In a particular embodiment of the first aspect or the second aspect of the invention, osteoblasts are obtained by collagenase digestion of living bone. The collagenase digestion is carried out by cleaning the bone of membranes and adherent tissue and

treating this with a mild trypsin solution, 1% trypsin in 0.05% EDTA in Puck's saline is suitable, and subsequently digesting the bone in a mild collagenase solution, such as 0.2% w/v collagenase type 2 (EC 3.4.23.3). It is preferred not to use the cells released by this digestion but to repeat the digestion to obtain second or, optionally, third stage digest osteoblasts which are suitable for use in the culture to form bone. The duration of digestion may vary according to the batch of collagenase and the source of cells; in a specific embodiment described below a 10 minute trypsin/EDTA digestion is used, and good results are obtained with a longer collagenase digestion period, specifically about 30 minutes or greater.

The invention also relates to the surface of culture apparatus or a culture plate per se. Accordingly, a third aspect of the invention provides apparatus for culture of osteoblasts, comprising a culture surface having topographical features that direct bone formation therein. The topographical features are preferably those described according to the first and second aspects of the invention, and also described in specific embodiments hereafter.

A particular culture plate of the invention comprises a plurality of spaced depressions adapted to enable or promote formation of bone therein. In a particular embodiment, the plate surface comprises a plurality of substantially parallel, substantially even-spaced grooves having depth in the range 100-500 microns and widths in the range 100-500 microns. A further embodiment of the third aspect of the invention comprises grooves having wall portions that meet substantially at a sharp angle, the angle being in the range 75-105°. By the term sharp angle it is intended to indicate that the wall portions meet at a sharp apex or at a junction that approximates to a radius of curvature of up to 50 microns.

A specific preferred embodiment of the invention includes grooves wherein wall portions meet substantially at 90°, these wall portions can be in a groove of substantially triangular, L shaped or rectangular cross section.

It is a further particular embodiment of the invention to provide a culture surface

wherein at least a portion of a wall of a groove is substantially normal to the plate surface, that is to say normal +/- 15°, and more preferably, where the plate is for use in a substantially horizontal position, a wall that is substantially vertical.

Methods of manufacture of suitable culture surfaces will be apparent to a person of skill in the art. The depressions, pits or grooves of the invention can be provided in the surface of a known culture surface, for example the surface of a petri dish, by for example cutting grooves therein with a sharp knife or other instrument. Suitable cutting instruments include diamond edged saws. Alternatively, if the plate is manufactured using a mould then this can be pre-formed to stamp out the depressions, pits or grooves of the invention into culture surfaces at the time of manufacture. The invention thus also relates to a method of modifying an osteoblast culture surface by providing therein depressions, pits and/or grooves according to any aspect of the invention.

In a fourth aspect, the invention relates to an implanted device on which is provided a surface according to the invention that is adapted to promote or enable bone formation thereon. By the term implanted device it is intended to include prosthetic devices such as surgical implants, bone grafts, and other implants made into or adjacent to bone.

The fourth aspect of the invention accordingly provides an implanted device having a surface adapted to promote or enable deposit and/or formation of bone thereon, the surface comprising a plurality of depressions. In an embodiment of this aspect of the invention the surface comprises a plurality of pits or grooves, or a mixture of pits and grooves. It is preferred that the pits or grooves have a width or diameter of 100-500 microns, and it is also preferred that the depth of the pits or grooves is in the range 100-500 microns. More preferred is a width in the range 250-450 microns, and a depth in the range 200-300 microns.

The invention thus provides an improved implant adapted to heal more rapidly following location into the recipient.

Metal and other materials are implanted into bone to restore lost function: common reasons are to replace knee or hip joints in patients suffering from osteoarthritis, to replace the head of the femur when the femoral neck has fractured in osteoporotic patients, to stabilise traumatic fractures and to replace lost teeth when the residual alveolar bony ridge is insufficient to support a denture. Bone is also transplanted in grafting operations.

An advantage of the invention is that by modifying the implant surface to encourage rapid bone formation, rapid healing is encouraged. This would obviate or reduce the need for cementation of implants. Cementless implants, if successful, may be difficult to remove for reorientation, but such reorientation might not be required if the healing process is directed and rapid.

The invention discloses a range of morphologies which will encourage bone growth, measuring the bone growth response in terms of the amount, quality and attachment of the bone, its rate of formation and expansion versus the shape (width, depth, radius of curvature) of features (grooves, pits) provided in otherwise flat surfaces. This has enabled development of an in vitro assay for appositional bone formation and the influence of pharmacological factors on such formation. This assay provides a new method for investigating the effect of any therapeutic agent on bone formation, including testing for side effects of a drug on bone formation and evaluating therapeutic agents for the treatment and/or prevention of osteoporosis and osteoarthritis.

However, at the present time, increasing numbers of other, e.g. skeletal and dental, implants are being placed in man and other mammals. The potential benefits that arise from this invention include faster healing, reduction in the suffering endured by patients having such implants and reduced operational risks.

Further potential benefit is in the manufacture of surgical tools (e.g. rotary milling tools) used in cutting live bone to shape the bed for an implant or graft, or the graft itself.

The invention is now exemplified by specific embodiments with reference to the accompanying drawings in which:-

Figs. 1, 2 and 3 show the results of an assay for the effect of 2 MeSATP on bone growth. In fig. 1 bone growth is measured by counting the number of bone loci in the grooves; in fig. 2 the area of bone growth in the grooves is measured; in fig. 3 the total length of bone formed in the grooves is measured; and

Fig. 4 shows the results of an assay for the effect of Stanozolol on bone growth.

Example 1: Bone Growth In Culture

We investigated the influence of substrate topography on the timing and location of bone formation by rat osteoblasts.

250μm thick slabs of dental tissues or compact bone were used intact or had a rectangular grid of grooves (ca.300μm wide and of variable depth) cut into the top surface with a diamond wheel. They were then seeded with osteoblasts harvested by enzyme digestion from rat calvarial bones from which all fibrous tissue had been removed previously. The cells were cultured in MEM (=minimum essential medium) with 10% FCS (=foetal calf serum) at 37^{0}C in 5% CO_2 for up to 4 weeks, and were observed daily alive. Ascorbic acid 50μg/ml and ß-glycero-phosphate 2mM were added at confluence (on the second or third day of culture). Specimens were fixed using 70% ethanol and stained with dilute alizarin to visualize the newly formed bone.

Bone formation began in the second week, preferentially wherever cellular condensation was favoured: these locations were a) the junction between the slab and the bottom of the culture dish, b) the periphery of the dish, c) cracks where dissimilar tissues had separated, and d) within the grooves. In the grooves, a grid of aligned bone developed, the deeper trenches showing bone formation earlier than shallower

ones, with bone formation tapering as a groove became shallower. Bone formation was initially found at the sharpest radius at the junction of the wall and floor of the trench in discrete nodules and spread linearly along the grooves. The cultures were maintained in some instances for 7 weeks and found to remain active throughout that time.

Example 2: Preparation Of Cells Which Will Form Bone In Culture

Osteoblastic cells are obtained from neonatal Sprague-Dawley rats by calvarial enzyme digestion in collagenase. The rat calvaria are excised and cleaned of membranes and adherent tissue before being placed in 1% trypsin/ 0.05% EDTA w/v in Puck's saline (Gibco) for 10 minutes at 37^{o}C followed by digestion in 0.2 % w/v collagenase type 2 (EC 3.4.24.3 derived from Clostridium histolyticum, Sigma) in PBS for 30 minutes or 1 hour @ 37^{o}C. Cells released at this stage are called 'first digest cells'.

The collagenase digestion stage as described above is repeated to obtain second and third digests.

In each case the cells released are centrifuged and re-suspended in, for example, Eagle's minimum essential medium with Earles salts, (EMEM, Sigma) containing 10% foetal calf serum (FCS, Gibco) and 2mM l-glutamine (Sigma) placed in culture flasks (Falcon) and grown in an incubator with a humidified 5% CO_2 atmosphere. When the cultures are approximately 80% confluent (at typically 3 days), the cells are trypsinized (1% trypsin/0.05% EDTA w/v in Puck's saline), centrifuged, re-suspended in EMEM, and counted by haemocytometer before being seeded on to the grooved substrates at a density of 200,000 cells/3.5cm Petri dish in EMEM supplemented with 10% FCS, and 2mM l-glutamine (Sigma) and incubated as above.

The cells are cultured for 2-3 days after which time the medium is replaced with EMEM supplemented with 10% FCS, 2mM l-glutamine, 50ug/ml l-ascorbic acid (Sigma, tissue culture grade), and 2mM B-glycerophosphate. The medium is

replenished every 2–3 days. On completion of the experiment, the slices are fixed and stored, for example in 70% ethanol @ 4°C, before measurement.

Example 3: In Vitro Bone Formation Assay

1. The assay assesses the amount of bone formed under standard culture conditions compared with that formed under test conditions.

2. The assay can be used either to test changes in the culture medium (such as by the addition of drugs, hormones or cytokines), or changes in the substratum (biocompatibility of materials, adhesion of appositional bone, coatings or proteins, etc.), or variations in bone cell populations.

3. Standard grooves 350μm across, 200μm deep, and of rounded cross section, are cut in thick slabs of dentine (bone or other mineralized tissue) or plastic coverslips or titanium alloys (or other material), or are impressed at the stage of manufacture of plastic tissue culture ware, such as in Petri dish bases or in the internal surfaces of tissue culture flasks.

4. Osteoblasts, obtained for example from rat cranial vault bones (second or third collagenous digest) are seeded on to grooved test slabs or dishes or flasks and the culture run for a typical period of up to 20 days, sometimes longer.

5. The culture is fixed, for example in 70% alcohol.

6. The specimen is stained with, for example, alizarin red S for 30 sec, washed in tap water, and the strain differentiated by storage in 70% ethanol.

7. The length of the groove(s) occupied by mineralized bone is measured and expressed as a ratio of the total length of groove(s).

Example 4: Bone Density

We examined the question whether bone formed by rat osteoblasts *in vitro* has a mineral density matching that of bone formed *in vivo*.

Rectangular grids of grooves, about 300 microns wide and of variable depth, were cut into 250 micron thick slabs of sperm whale dentine with a diamond wheel. The slabs were seeded with primary rat calvarial osteoblasts and cultured in MEM with 10% FCS at 37°C with 5% CO_2. Ascorbic acid 50μg/ml and ß-glycero-phosphate 2mM were added at confluence. Cultures were observed daily until fixed in 70% ethanol at times from 2–4 weeks, when they were embedded in poly-methyl-methacrylate (PMMA): block-faces were micromilled, coated with carbon and examined by digital backscattered electron (BSE) imaging. Reference specimens were PMMA embedded rat bone (15 week femoral shaft and 2 year old mandible) and halogenated dimethacrylate ester standards. The proportion of bone or dentine falling in 8 equal bins in the range from $C_{22}H_{25}O_{10}Br$ (mean BSE coefficient = 0.1159, scaled to 0) to $C_{22}H_{25}O_{10}I$ (mean BSE coefficient = 0.1519, scaled to 255) was calculated. A grid of cellular bone developed in the grooves, and bone formation ringed the dentine slab. The new bone was commonly attached at the sharply radiussed part of the deeper trenches first as small discrete nodules which then extended as ribbons along the grooves, tapering off as the grooves became shallower. The peak mineral density of the bone formed vitro was in bin 7; 34% of *in vitro* bone was in this bin as against 45% of *in vivo* bone and 67% of dentine. 25% of *in vitro* bone, <10% of *in vivo* bone and only 1% or less of the dentine, fell to the lower 5 bins. We conclude that the bone formed by rat osteoblasts *in vitro* attains the same mineral density as that formed *in vivo*.

Example 5: Metal And Plastic Substrates

We examined the question whether the new appositional bone formation would occur equally with metal and plastic substrates.

We cut grooves and pits in (a) titanium alloy slices prepared from retrieved implants (previously used in vivo in man or rabbit) and (b) the bases of plastic tissue culture dishes, and seeded them with rat calvarial second collagenase digest cells (as above). New bone formation was observed to occur first in the surface depressions in both the Ti alloy and the plastic substrates.

New bone formation in the transparent plastic substrates could be seen without staining, and with enhanced clarity after alizarin staining.

We observed that the alizarin stained new bone in grooves in the (opaque but reflective) metal substrates could be imaged to very good effect in the reflected light mode by using a video camera coupled to an imaging fibre optic bundle, giving an enlarged TV image on a colour TV monitor. The proprietary system used was the Moritex Scopeman 504 with a lens giving a screen magnification of 25X, but any of several similar devices could have been used with equal convenience.

Example 6: 2 MeSATP Assay

The assay of Example 3 was used to test the effect of the ATP analogue 2 MeSATP on bone growth. The method was as set out in Example 3 using 2 MeSATP at 0, 2 and 20μM. Assay results were measured as number of bone loci formed/SWD slice (fig.1), mean bone area on a dentine slice ($\mu m^2 \times 10^{-3}$) (fig. 2) and mean length of bone formed (mm) (fig. 3). In fig. 1 *=$p<0.002$ (t-test), in fig.2 *=$p<0.003$ (Mann-Whitney) and in fig. 3 *=$p<0.002$ (Mann-Whitney).

Example 7: Growth Of Previously Frozen Cells

Example 1 was repeated using osteoblasts that were frozen and recovered from frozen storage according to standard techniques. No noticeable change to the pattern or amount of bone growth was seen compared with the control.

Example 8: Stanozolol Assay

The assay of Example 3 was used to test the effect of Stanozolol, using concentrations of 5×10^{-7}M, 5×10^{-8}M and 5×10^{-9}M and a control (no Stanozolol). An enhancement of bone growth compared with the control (fig. 4) was observed but did not attain statistical significance (at p= 0.05).

Example 9: PTH Assay

The assay of Example 3 was used to test the effect of human PTH and rat 1-34 PHT. Human PTH was used at concentrations of 0.25 and 0.5 International units and 1-34 PTH was used at 10^{-7}M and 10^{-8}M, with the usual controls (no PTH).

Both human PTH and 1-34 PTH showed strong inhibition of bone growth, in that no bone growth was detectable even at the 28th or 29th day of culture, whereas bone growth was detected in the controls after about 18 days-details are shown in the table below:-

Table I

		Mineralised Bone Formation
Human PTH	0	6/6 at 18 days, 1/1 at 29 days
	0.25 I.U.	0/6 at 18 days
	0.50 I.U.	0/6 at 18 days, 0/1 at 29 days
Rat 1-34 PTH	0	growth seen at 19 and 28 days
	10^{-8}M	no growth at 19 nor 28 days
	10^{-7}M	no growth at 19 nor 28 days

The following are examples of industrial application of this invention.

*) For skeletal and dental implants: controlling the loci at which new bone formation attached to an implant may occur, by (a) encouraging new growth by the means considered here at intervals corresponding to the intervals required between trabecular rods and/or plates, such intervals derived by analogy with structures found in natural bone or by finite element stress–strain modelling techniques, and (b) by preventing bone tissue attachment to the implant surface in intervening, predefined regions by application of surface coating materials such as polytetrafluoroethylene (Teflon) or any other material having poor cell–adhesion properties, we claim the means to control the distribution of load in the layers closest to the implant, leading to means to control to some extent the extent of stress shielding.

*) For skeletal and dental implants: manufacturing implants with surface features which encourage the rapid formation of new bone, thus aiding the healing process.

*) For laboratory exploitation: developing tissue culture systems to control the sites of development of new bone tissue, thereby permitting the study of all stages in the development of bone by exploiting the prior knowledge that new bone will form at a particular loci; for example, by manufacturing tissue culture plastic or glassware with appropriately embossed or indented features.

*) For operation on bone sites in the preparation for implants: designing new rotary bone burs or rasps with cutting blades designed to produce a surface profile in the cut bone such as will encourage rapid and effective healing with good quality, new bone.

CLAIMS

1. A method of promoting or enabling bone formation in vitro comprising culturing osteoblasts on a surface having topographical features that direct said formation in a localized area.

2. A method according to Claim 1 wherein a first surface portion is depressed in relation to an adjacent, raised surface portion.

3. A method according to Claim 2 wherein there is a substantially L or U or semi-circular or V shaped concave junction between the respective depressed and raised surface portions.

4. A method according to any of Claims 1-3 wherein the surface comprises one or a plurality of grooves or pits adapted to promote or enable bone formation.

5. A method according to any of Claims 2-4 wherein a depression in the surface is, in cross-section, substantially U shaped, triangular, rectangular or semi-circular.

6. A method according to any of Claims 2-5 wherein a depression in the surface has a width of 50-500μm.

7. A method according to any of Claims 2-6 wherein a depression in the surface has a depth of 100-500μm.

8. A method according to any preceding claim comprising harvesting bone from the culture.

9. A method of assaying a substance for its effect on bone formation by osteoblasts, comprising:-

(a) culturing osteoblasts in the presence of the substance according to the

method of any one of Claims 1-7.

10. A method according to Claim 9 comprising comparing results obtained from step (a) with results obtained from a similar culture in the absence of the substance.

11. A method according to Claim 9 or 10 wherein osteoblasts are cultured on a surface comprising a groove and wherein along at least a portion of the groove its depth gradually decreases.

12. A method according to Claim 11 wherein along a portion of groove its depth varies between 200 μm and 300 μm.

13. Apparatus for in vitro culture of osteoblasts comprising a culture surface having topographical features according to any of Claim 1-7.

14. A plate surface for culture of osteoblasts comprising a plurality of depressions, pits or grooves with depths of 50-500 μm and widths of 100-500 μm.

15. A plate surface according to Claim 14 wherein the groovés are substantially rectangular in cross section.

16. A plate surface according to Claim 14 wherein the grooves are substantially triangular, V shaped or semi-circular in cross section.

17. A plate surface according to any of Claims 14-16 wherein the grooves include walls portions that meet at an angle of 75-105°.

18. A plate surface according to Claim 17 wherein the wall portions meet substantially at 90°.

19. A plate surface according to Claim 14 wherein the grooves are substantially rounded in cross section.

20. A plate surface according to any of Claims 14–19 wherein at least a portion of a wall of the grooves is substantially normal to the plate surface, that is to say +/– 15°.

21. An implanted device comprising a surface having topographical features to enable or promote bone deposit and/or formation thereon, said features comprising a plurality of depressions.

22. An implanted device according to Claim 21 having a surface adapted to enable or promote deposit and/or formation of bone thereon, the surface comprising a plurality of pits or grooves, or a mixture of pits and grooves.

23. A device according to Claim 22 wherein the pits or grooves have a width or diameter of 100–500 μm and a depth of 50–500 μm.

24. A device according to Claim 22 or 23 wherein the pits or grooves include a junction between walls thereof having an angle of 75–105°.

25. A device according to Claim 24 wherein the apex of the junction between the walls is a sharp apex or approximates to a radius of curvature of up to 50 μm.

26. A device according to any of Claims 22–25 wherein the pits or grooves include at least one wall substantially normal to the device surface, that is +/– 15 degrees.

27. A device according to any of Claims 21–26 having a bone-growth or bone deposition promoting surface comprising a plurality of grooves, spaced from each other and cut into or otherwise provided in the surface, the grooves being substantially rectangular or substantially V shaped, triangular or substantially rounded in cross section, and having a depth in the range of 150–500 μm and a width in the range of 100–500 μm.

28. A device according to any of Claims 21–27 wherein a second surface of the device is adapted to prevent or deter bone growth or bone deposit thereon.

29. A device according to Claim 28 wherein said second surface is coated with a bone growth or bone deposit inhibitor.

30. A method of modifying a surface of an implanted device, comprising:-

(a) providing in said surface a plurality of topographical features adapted to enable or promote deposit and/or formation of bone thereon, said features comprising one or a plurality of pits or grooves.

31. A method according to Claim 30 wherein the width of pits or grooves is 100-500 μm.

32. A method according to Claim 30 or 31 wherein the depth of pits or grooves is 50-500 μm.

33. A method of modifying a surface of a bone (1) prior to transplant thereof, (2) prior to receipt of a transplant thereon, or (3) prior to receipt of a prosthetic device thereon, comprising:-

(a) providing in said surface a plurality of topographical features adapted to enable or promote deposit and/or formation of bone thereon, said features comprising one or a plurality of pits or grooves or pits.

34. A method according to Claim 33 wherein the width of pits or grooves is 100-500 μm.

35. A method according to Claim 33 or 34 wherein the depth of grooves or pits is 50-500 μm.

36. A method of modifying a surface of a prosthetic device comprising:-

(a) modifying a first area of the surface to promote growth or deposit of bone thereon, according to any of Claims 21-27, and

(b) modifying a second area of the surface to inhibit growth or deposit of bone thereon.

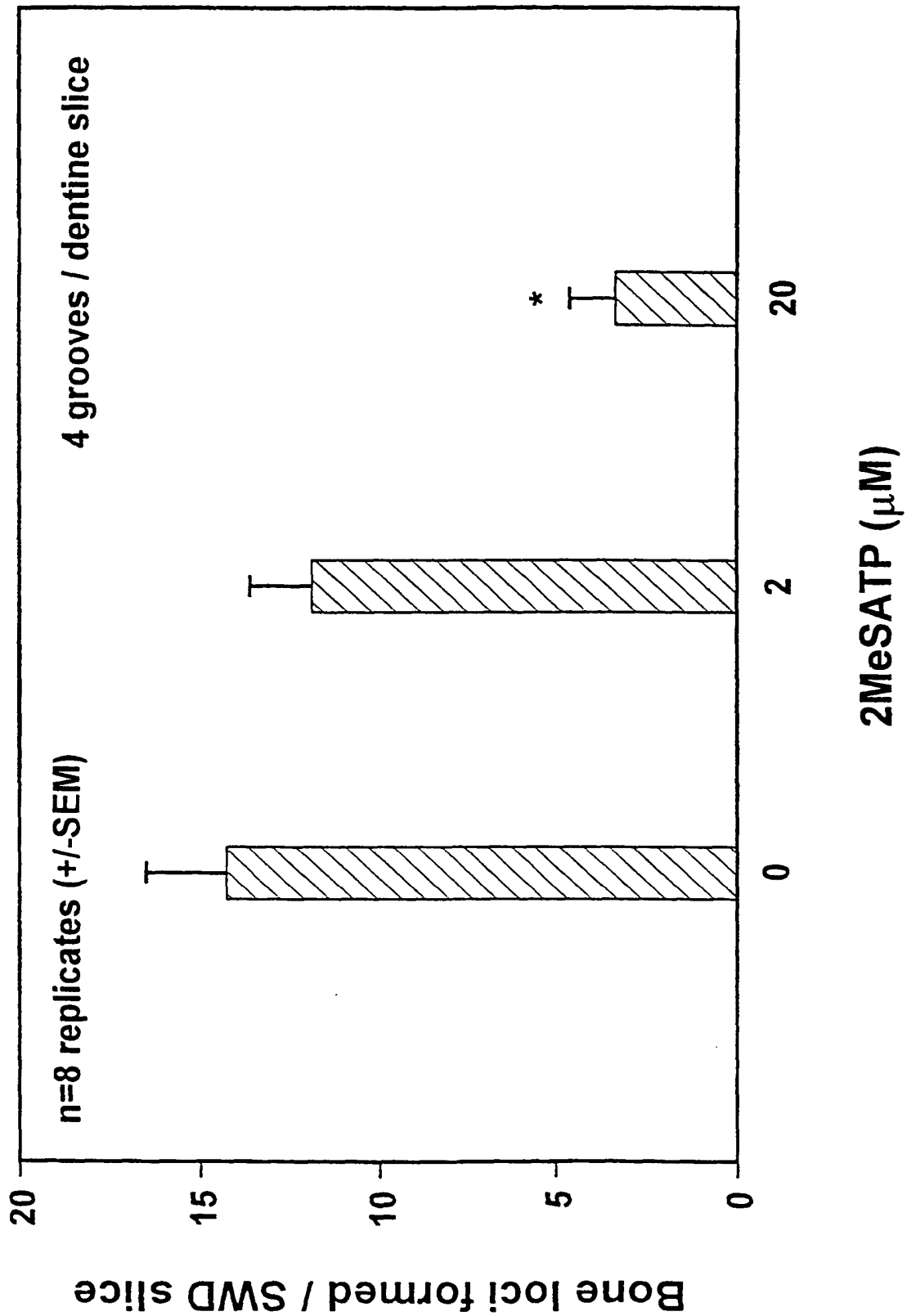


Fig. 1

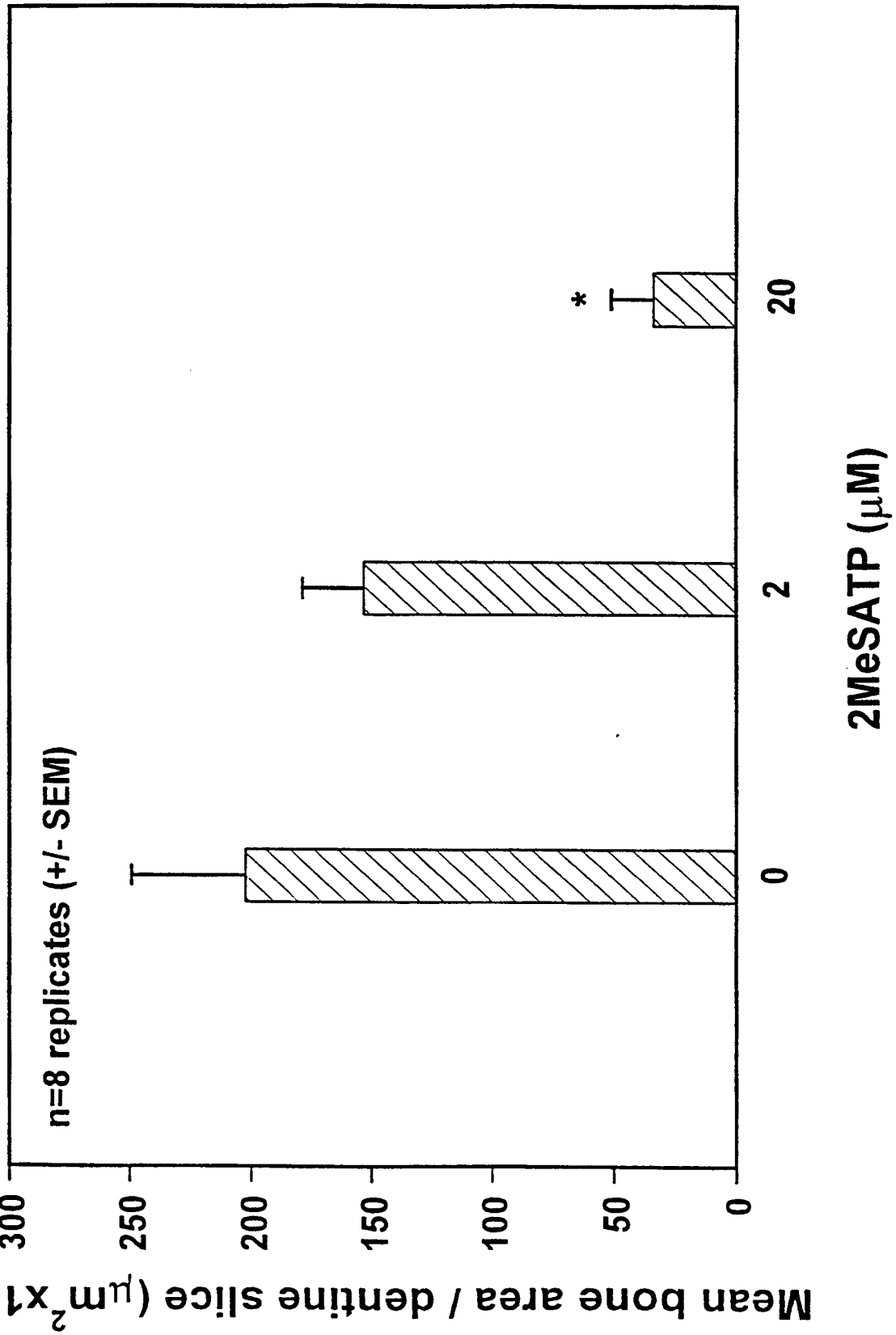


Fig. 2

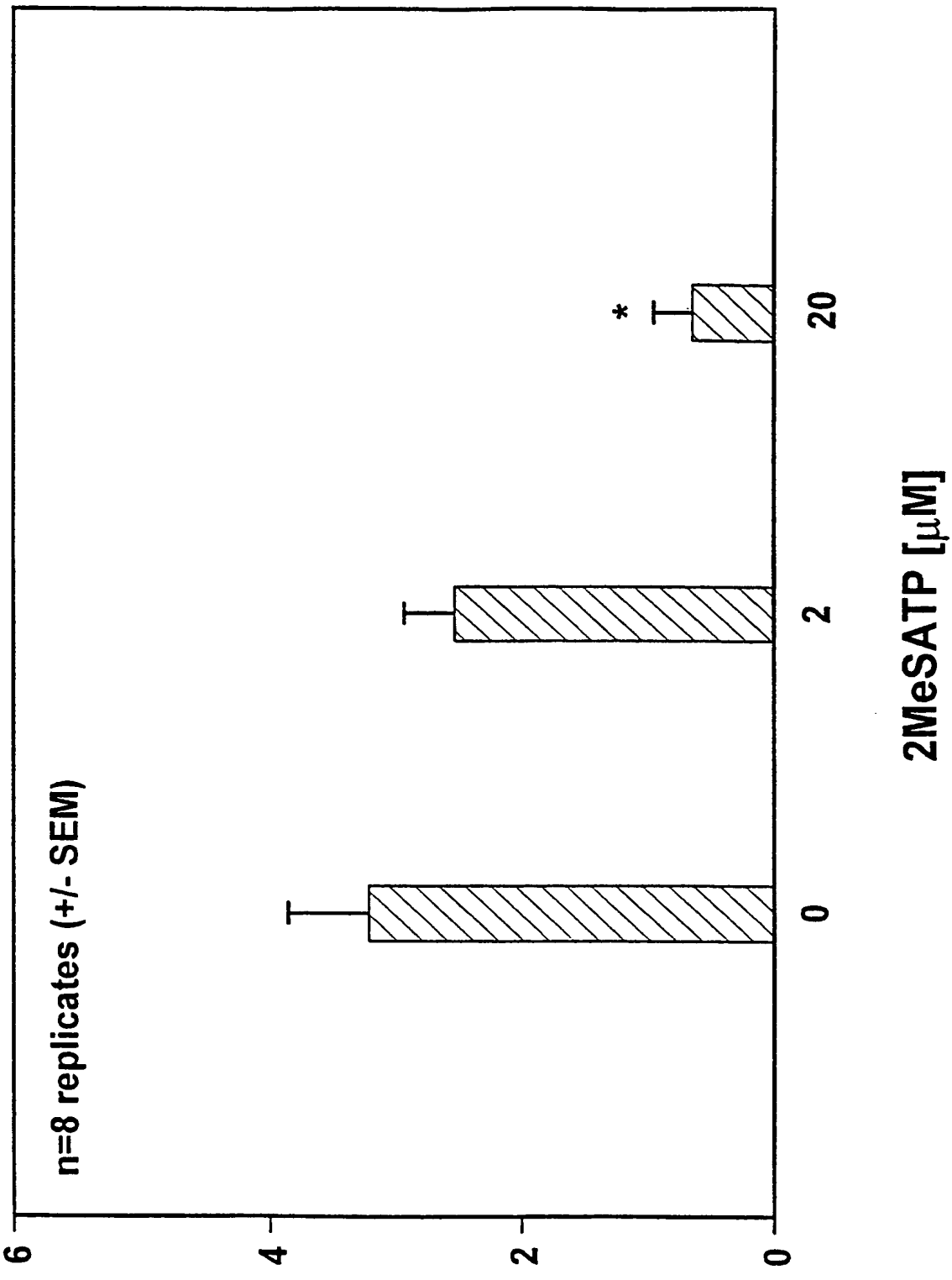
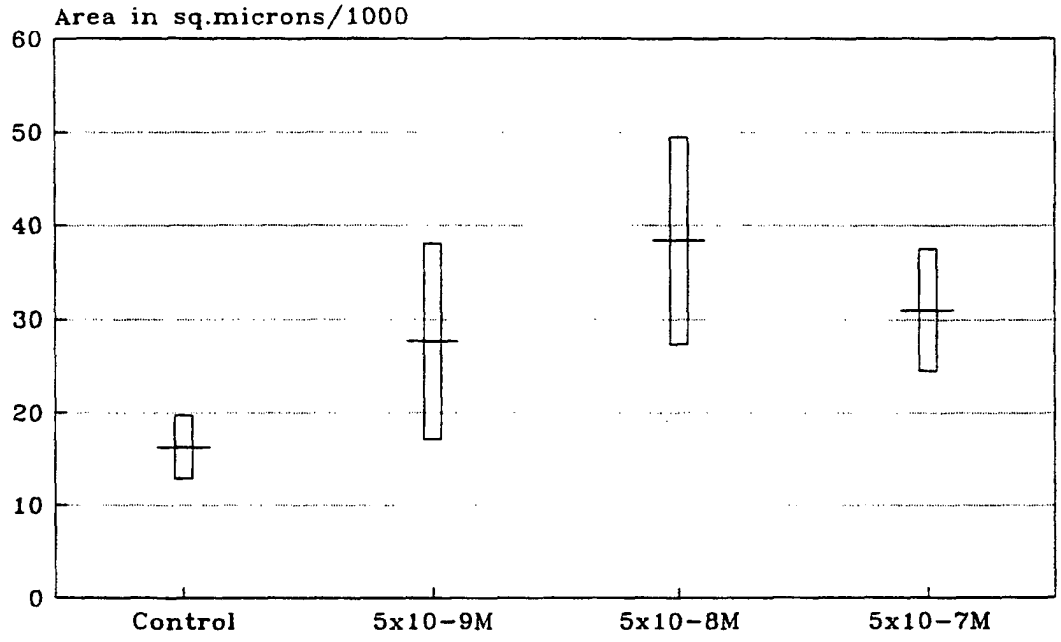


Fig. 3



Rat osteoblasts on grooved dentine
20 days in vitro: Means and S.E.M.

Fig. 4

INTERNATIONAL SEARCH REPORT

Int[redacted] Application No
PCT[redacted]96/01818

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00 A61L27/00 C12M3/04 C12Q1/02 C12M1/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61L C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 04657 (UNIV PENNSYLVANIA) 3 March 1994 see the whole document ---	1-36
X	DE,A,41 32 379 (KERNFORSCHUNGSZ KARLSRUHE) 8 April 1993 see example 1 ---	13-16, 20,30-32
X	GB,A,2 262 538 (CORNING INC) 23 June 1993 see the whole document ---	13-20, 30-32
X	US,A,5 112 354 (SIRES BRYAN S) 12 May 1992 see the whole document ---	21-34
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 October 1996	25. 10. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Fernandez y Branas, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	BIOMATERIALS, vol. 10, no. 1, 1989, pages 63-67, XP000604999 CHEUNG H.S. ET AL: "Growth of osteoblasts on porous calcium phosphate ceramic: an in vitro model for biocompatibility study" see the whole document	13-20
X	CLINICAL ORTOPAEDICS AND RELATED RESEARCH, vol. 262, 1991, pages 298-311, XP002015230 GOSHIMA J. ET AL: "The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed in vivo in calcium phosphate ceramic blocks" see the whole document	21-27, 30-35
X	EP,A,0 175 286 (MCW RESEARCH FOUNDATION INC) 1986 see page 16, line 14 - line 24 see example 5 see page 31, line 27 - line 37	1-6,13
A	WO,A,95 02687 (MAYO FOUNDATION ;HARRIS STEVEN A (US); SPELSBERG THOMAS C (US)) 26 January 1995 see the whole document	9-13
A	WO,A,89 06945 (BIOMEDICAL DESIGN INC) 10 August 1989 see the whole document	28,29,36

INTERNATIONAL SEARCH REPORT

In... application No.
PCT/GB96/01818

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
please see annex

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/01818

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